PHYSICOCHEMICAL PROFILING AND DETECTION OF PHENOLIC CONSTITUENTS WITH ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *Myristica fragrans* HOUTT.

by

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To my late grandmother, Hajah Siti Rafeah bt Haji Ahmad

ا لفاتحه

Nutmeg in literature

I had a little nut tree,
Nothing would it bear,
But a silver nutmeg,
And a golden pear.

The King of Spain's daughter,
Came and visit me,
And all for the sake,
Of my little nut tree.

I skipped over ocean,
I danced over sea,
And all the birds in the air,
Couldn't catch me.

Bismillahirrahmanirrahim

(In the name of Allah, The Most Gracious, Most Merciful)

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ABSTRAK

Penyelidikan ini dijalankan untuk menilai ciri fizikal (warna) dan kimia (analisis proksimat dan mineral) serta aktiviti antioksidan dan antibakteria ekstrak daun, perikarpa, aril, biji, isi tempurung dan tempurung *Myristica fragrans* Houtt. Analisis warna menunjukkan bahagian aril memiliki nilai *a** dan *C* yang tinggi (28.14 ± 0.49 dan 29.92 ± 0.58, masing - masing) dengan sudut hue (h^o) paling rendah (19.87 ± 0.35^o), kulit perikarpa pula mencatatkan nilai *b** paling tinggi (28.59 ± 0.78) dan isi tempurung mencatatkan parameter *L** paling tinggi (72.85 ± 0.16). Kandungan lembapan adalah paling tinggi untuk semua sampel kecuali tempurung. Sementara itu, kesemua sampel memiliki kandungan abu yang rendah kecuali bahagian daun dan isi tempurung, di mana kandungan proteinnya lebih rendah berbanding abu. Analisis unsur utama (Ca, Na, K, Mg) dan unsur surih (Cu, Mn, Fe, Zn) menunjukkan kepekatan kalium (K) dan kalsium (Ca) yang tertinggi dalam perikarpa manakala mangan (Mn) merupakan unsur surih paling tinggi dikesan. Ekstrak mempamerkan julat kandungan fenolik total yang luas daripada 649.00 ± 2.16mg GAE/g ekstrak kering hingga 8.66 ± 0.71mg GAE/g ekstrak kering. Aktiviti antioksidan berbeza daripada 84.53 ± 0.89% hingga 12.57 ± 0.98%. Korelasi yang signifikan dan positif telah direkodkan bagi kandungan fenolik total dan aktiviti antioksidan (r^2 = 0.7039, p < 0.0001). Ini menunjukkan bahawa fenolik merupakan bahan antioksidan utama di dalam ekstrak. Sejumlah lapan fraksi diperolehi dari

ekstrak krud aseton 70% bahagian daun dan af4 mempamerkan nilai terbaik bagi asai antioksidan (nilai EC_{50} = 24.91 ± 0.29µg/mL) dan paling tinggi kandungan fenolik total (526.68 ± 0.82mg GAE/g ekstrak kering). Sub - fraksi af4i pula menunjukkan nilai terbaik untuk aktiviti antioksidan (nilai EC_{50} = 23.08 ± 0.61µg/mL) dan sub - fraksi af4iii mengandungi kandungan fenolik total yang paling tinggi (579.05 ± 0.46mg GAE/g ekstrak kering). Daripada lapan fraksi dari kromatografi turus bagi ekstrak aglikon aseton 70% bahagian perikarpa, cf2 mempamerkan nilai terbaik untuk kedua - dua kandungan fenolik total (358.85 ± 1.32mg GAE/g ekstrak kering) dan asai antioksidan (nilai EC_{50} = 38.91 ± 1.81µg/mL). Aktiviti antibakteria oleh kesemua ekstrak adalah lebih menonjol ke atas bakteria Gram - positif berbanding bakteria Gram - negatif. Diameter zon perencatan bagi ekstrak berada dalam julat 16.00 ± 0.00mm hingga 9.00 ± 0.00mm. MFa10 yang disisihkan daripada ekstrak aseton 70% biji adalah juzuk yang paling poten dengan nilai MIC 37.50µg/mL dan nilai MBC 150.00µg/mL terhadap *Staphylococcus aureus* ATCC 12600. Empat komponen fenolik iaitu kuersetin, kuersetin - 3 - *O* - glukosida, skandinon dan sebatian lignan yang tidak dikenalpasti telah disisihkan dan dicirikan sebagai juzuk utama dengan aktiviti menyingkirkan radikal bebas dan antibakteria.

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ABSTRACT

This study was conducted to evaluate physical (color) and chemical (proximate and mineral analysis) characteristics as well as the antioxidant and antibacterial activity of extracts from leaves, pericarps, maces, seeds, seed kernels and shells of *Myristica fragrans* Houtt. The color analysis revealed that the mace has the highest a^* and C value (28.14 ± 0.49 and 29.92 ± 0.58, respectively) and the least hue angle (h^o) (19.87 ± 0.35^o), skin of pericarp was detected to have the highest b^* value (28.59 ± 0.78) and seed kernel exhibited the highest L^* parameter (72.85 ± 0.16). Moisture content was at the highest for all samples except for shell. Meanwhile, all samples were low in ash content except for leaf and seed kernel, whereby their protein contents were lower than ash. Analysis for the major elements (Ca, Na, K and Mg) and for the minor and trace elements (Cu, Mn, Fe and Zn) showed the highest concentration of potassium (K) and calcium (Ca) in the pericarp while manganese (Mn) is the predominant microelement detected. These extracts exhibited a wide range of total phenolic content varying from 649.00 ± 2.16mg GAE/g dry extract to 8.66 ± 0.71mg GAE/g dry extract. The antioxidant activity varied from 84.53 ± 0.89% to 12.57 ± 0.98%. Significant and positive linear correlation were recorded for total phenolic content and antioxidant activity (r^2 = 0.7039, p < 0.0001), indicating that phenolics were the major antioxidant constituents in the extracts. A total of eight fractions were collected from 70% acetone crude extract of the leaf and

af4 exhibited the greatest value in antioxidant assay (EC_{50} value = 24.91 ± 0.29μg/mL) and the highest in total phenolic content (526.68 ± 0.82mg GAE/g dry extract). Sub - fraction af4i showed the greatest value in antioxidant activity (EC_{50} value = 23.08 ± 0.61μg/mL) and sub - fraction af4iii was the highest of total phenolic content (579.05 ± 0.46mg GAE/g dry extract). From eight column chromatographic fractions of the aglycone of 70% acetone extract of the pericarp, cf2 showed the greatest values in both total phenolic content (358.85 ± 1.32mg GAE/g dry extract) and antioxidant assay (EC_{50} value = 38.91 ± 1.81μg/mL). The antibacterial activity of all the extracts was more pronounced against Gram - positive bacteria than Gram - negative bacteria. The inhibition zone diameters of extracts were ranging from 16.00 ± 0.00mm to 9.00 ± 0.00mm. MFa10 that was purified from 70% acetone extract of the seed was the most potent component with MIC value of 37.50μg/mL and MBC value of 150.00μg/mL against *Staphylococcus aureus* ATCC 12600. Four phenolic substances, which were quercetin, quercetin - 3 - *O* - glucoside, scandinone and an unidentified lignan compound were purified and characterized as the main constituents with free radical scavenging and antibacterial activities.

CHAPTER 1 INTRODUCTION

1.1 *Myristica fragrans* Houtt.

1.1.1 Morphological description

Myristica fragrans Houtt., locally known named *pala* in Malay is an evergreen tree growing to a height of about 18m (Ong, 2004). It belongs to the Myristicaceae family. The leaves are oval in shape, pinnately and alternately arranged with fragrance odor when crush (Weiss, 2002). The aromatic flowers are pale yellow in color and are clustered in sima umbellate inflorescence (Weiss, 2002; Ong, 2004). *M. fragrans* produces drupe type fruits, pyriform in shape, 6cm to 9cm long, yellowish skin with perpendicular groove around the fruit and whitish flesh (Weiss, 2002). The flesh is about 1.3cm thick and contributes 75% to 85% of total weight. It splits when ripe revealing its red mace encasing its brown glossy seed (Felter & Lloyd, 1898). Parts of *M. fragrans* fruit are shown in Plate 1.1.

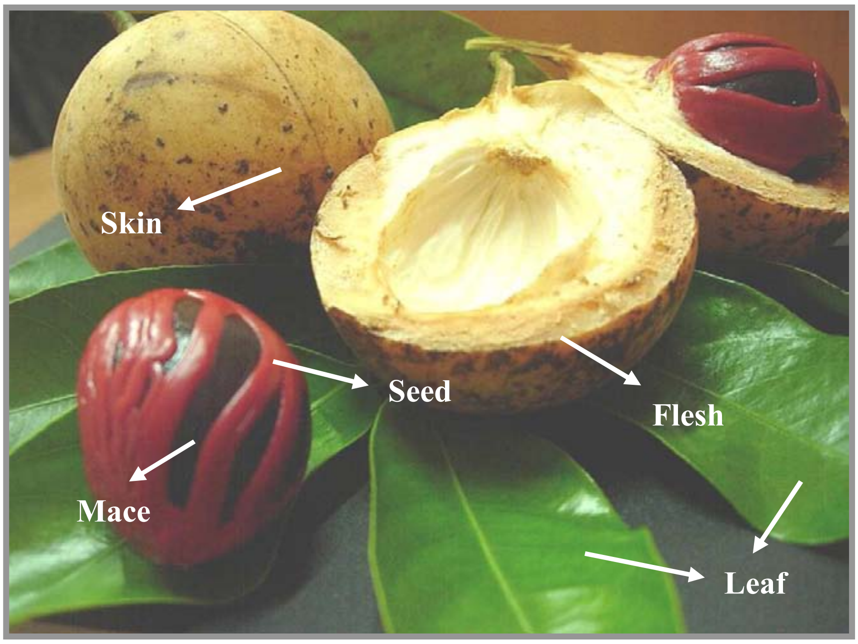

Plate 1.1 *Myristica fragrans* Houtt.

1.1.2 Therapeutic values

The leaves are drunk as tea to relief flatulence and intestinal spasm (Flach & Willink, 1999). The shoots are also used medicinally to treat hypertension (Mustapa, 2008). The mace was also used as stomach tonic and for healing headache and migraine (Zaidi *et al*., 2009). In Indonesian folk medicine the mace is used for curing rheumatism. The seed kernel (nutmeg) is widely used as spice with possible health beneficial effects such as aphrodisiac, anthelmintic, anticonvulsant and antiseptic. It is also useful in treating inflammation, vomiting, diarrhea, dysentery, asthma, heart disease, liver and spleen disorder, insomnia, colic, menorrhagia (Sharma *et al*., 2002), flatulence, nausea and dyspepsia (Zaidi *et al*., 2009). A resin obtained from the bark is applied externally to treat polyarthritis and gout (Adams *et al*., 2009).

In addition to its medicinal uses, the sour ripe fruits are used for preparing pickles, jams, sweets and jellies while the seed and mace are used as flavoring for fish, meat, biscuits, cakes, sauces and soups (Ong, 2004).

1.1.3 Scientific biological and pharmacological studies

Various biological and pharmacological evaluations have been conducted to verify the therapeutic values of *M. fragrans*. The ethanolic extract of the pericarp exhibited hypolipidaemic effect by lowering the total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides levels in the treated albino rabbits after 60 days. In addition, the extract also showed platelet anti - aggregatory effect with no side effects on various hematological and biochemical parameters (Ram *et al*., 1996).

Meanwhile, Ozaki *et al*. (1989) have reported the anti - inflammatory effect of methanol extract of the mace and myristicin was detected as the active constituent. Jannu *et al*. (1991) have tested the effectiveness of mace as chemotheraphy agent on 7, 12 - dimethylbenzanthracene (DMBA) - induced papillomagenesis in the skin of male albino mice. The extract reduced 50% of papillomagenesis (as compared with that of control (100%)). Hussain and Rao (1991) reported that at the dose of 10mg/mouse/day orally for seven days before and ninety following days, mace exhibited good chemopreventive activity by reducing the cervical carcinoma incidence from 73.90% (control) to 21.40%. Moreover, Kumari and Rao (1989) reported the hepatoprotective effect of the mace based on a significant increase in gluthathione - S - transferase (GST) and acid soluble sulfhydryl (SH) levels.

Another studied conducted by Sharma and colleagues (1995) demonstrated the efficacy of seed extract as preventive agent for hypercholesterolemia and atherosclerosis in rabbits. The extract assisted in reducing serum cholesterol, LDL cholesterol and cholesterol/phosholipid ratio by 69.10%, 76.30% and 31.20% respectively and also significantly elevated the decrease of high density lipoprotein (HDL). Moreover, the extract also prevented the accumulation of cholesterol, phospholipids and triglycerides in liver, heart and aorta, dissolved the atheromatous plaques of aorta from 70.90% to 77.65% and increased the fecal excretion of cholesterol and phospholipid. Furthermore, Olajide *et al*. (1999) found that the chloroform extract of the seed showed anti - inflammatory activity by inhibiting the rat paw oedema. The extract also has analgesic property by reducing writhings. Sonavane *et al*. (2001) reported that the *n* - hexane extract of the seed has anxiogenic, sedative and analgesic activities. The results obtained from Parle *et al*. (2004) revealed that the *n* - hexane extract of the seed at lowest dose of 5mg/kg administered for three days, improved learning and memory of young and aged mice. This extract also reversed scopolamine - and diazepam - induced impairment in learning process and memory. Goncalves *et al*. (2005) found that the aqueous seed extract was able to inhibit only human rotavirus cell (HCR3) with 90.00% inhibition at the maximum non - toxic concentration (MNTC) of 160.00µg/mL.

The crude suspension and petroleum ether extract of seed kernel possessed a good antidiarrheal effect and sedative property, with weak analgesic effect (Grover *et al*., 2002). An experimental study by Tajuddin *et al*. (2003) and Tajuddin *et al*. (2005) were undertaken to evaluate the improving effect of 50% ethanolic extract of the seed kernel on sexual function. The result indicated that the extract increased both libido and potency, which might be attributed to its nervous stimulating property. Apart from possessing aphrodisiac effect, the extract was observed to be devoided of any adverse effects and acute toxicity. Meanwhile, Janssens *et al*. (1990) suggested that eugenol and isoeugenol (the major components of seed kernel oil) play the major role in the inhibiting platelet aggregation. Moreover, based on the plasma aminotransferase activities, the seed kernel oil and myristicin showed a prominent hepatoprotective activity (Morita *et al*., 2003). According to antidepressant study conducted by Tan (2006) via tail suspension test (TST) and forced swimming test (FST), the hexane, chloroform and 80% methanol extracts of pericarp, mace, kernel and seed generally showed poor antidepressant effect.

Apart to its health beneficial effects on human, *M. fragrans* do play a role as insect controlling agent. The results obtained from Huang *et al*. (1997) suggested that the essential oil of the seed extract exhibited good grain protectant property as the oil reduced the production of progeny of *Tribolium castaneum* and *Sitophilus zeamais*, decreased in the percentage of eggs hatched and viable larvae after hatching at various concentrations.

1.1.4 Phytochemical content

The identified constituents based on spectroscopic methods reported by numerous authors were compiled in Table 1.1. As indicated in the Table, pericarp, mace and seed kernel share constituents, such as α - pinene, β - pinene, limonene and sabinene. Choo *et al*. (1999) asserted that the constituents present are similar in pericarp, mace and seed kernel though the compositions are substantially different. As far as literature survey could as certain, no report on chemical composition of essential oil and extract of shell *M. fragrans* was obtained.

Table 1.1 Comparison of constituents in *M. fragrans*

Plant part	Constituents
Leaf	myristicin, quercetin and kaempferol (Suhaj, 2006).
Pericarp	α - pinene, β - pinene, γ - terpinene, α - terpinene, α - terpineol, myristicin, limonene, sabinene, α - terpinolene, α - myrcene, terpinen - 4 - ol, isoeugenol, myristicin (Choo *et al*., 1999), ferulic acid (Wojdylo *et al*., 2007), caffeic acid, catechin (Shan *et al*., 2005), quercetin, and kaempferol (Suhaj, 2006).
Mace	α - pinene, β - pinene, limonene, safrole, sabinene, lignans, neolignans (Hada *et al*., 1988), linoleic acid, palmitic acid, elemicin, isocroweacin, methoxyeugenol, isoeugenol (Singh *et al*., 2005), cyanidin, quercetin and kaempferol (Suhaj, 2006).
Seed	isoeugenol, methyl-eugenol, eugenol, dihydroguaiaretic acid, γ - terpinene, terpinen - 4 - ol, myristic acid, oleanolic acid, palmitic acid, camphene, lauric acid, myrcene, quercetin and kaempferol (Suhaj, 2006)
Seed kernel	α - pinene, β - pinene, sabinene, safrole, terpinen - 4 - ol, elemicin, myristicin, α - terpineol, myristicin, limonene, α - terpinene (Spricigo *et al*., 1999; Tomaino *et al*., 2005; Jukić *et al*., 2006), eugenol, isoeugenol (Janssens *et al*., 1990), neolignans (myrisfragransin) (Li & Yang, 2008), lignans (diarylbutane, 7 - methyl ether diarylbutane and aryltetralin) (Kwon *et al*., 2008), neolignan (myrislignan), macelignan (Chung *et al*., 2006).

1.2 Nutritional aspect

There are seven major classes of nutrients which are carbohydrates, fats, fiber, minerals, proteins, vitamins, and water (Watkin, 1979). These nutrients can be generally grouped into macronutrients and micronutrients (Whitney & Rolfes, 1996). The macronutrients are carbohydrates, fats, fiber, proteins and water. On the other hand, minerals and vitamins are called micronutrients. Minerals can be divided into two groups. First group is macroelement (calcium, phosphorus, potassium, sodium and sulfur) which are required in large quantities above 100mg/kg diet. Second group is microelements (ferum, cuprum, manganese, zinc, cobalt, molybdenum, chromium, selenium, flour, iodine and nickel) which are required in small amount below 100mg/kg diet.

Primarily, carbohydrates, proteins, and fats are metabolized to give energy. Protein serves as the major structural component of all cells in the body, and functions as enzymes, in membranes, as transport carriers, and as some hormones. Minerals are essential chemical elements in human body which are involved in the formation of skeletal structure, blood protein, enzymes and hormones, maintenance of colloidal system and regulation of acid base equilibrium. They also act as component which involved in enzyme activation, hemoglobin composition and lipid, amino acid and carbohydrate metabolism (Mason, 2001).

Plants contribute notably to human nutrition and health, because they contain almost all essential mineral and organic nutrients. Nutrient composition varies among different plants' parts and species (Sanchez - Castillo *et al*., 1998) and not all plants contain essential nutrients needed for individual health. For instance, leafy vegetables are good sources of most minerals and vitamins with less concentration of protein and carbohydrates. Seeds are good sources of carbohydrates, proteins, lipids, and lipid - soluble vitamins, but tend to have low concentrations of iron and calcium (Grusak & DellaPenna, 1999). To ensure an adequate dietary intake of all essential nutrients and to increase the consumption of various health - promoting plants, it is an urgent need for researchers to quantify and compile all the nutritional information of all food plants (Arzani *et al*., 2007).

The human body requires a number of minerals in order to maintain good health. Malnutrition is major nutrition concern for tropical countries. Malnutrition is a concept of nutrition disorder. The disorder may be due to excessive nutrition (overnutrition) or deficiency nutrition (undernutrition) (McLaren, 1979). In the developing world, many low - income families survive on a simple diet comprised primarily of staple foods such as rice, wheat and maize that are poor in some macronutrients and many micronutrients. As a result, 30% of the world's population is at risk for iron deficiency anemia (infants, children, and women at reproductive age are particularly vulnerable) (Arzani *et al*., 2007). Hardisson *et al.* (2001) reported that the risk of deficiencies depends on a number of factors such as the daily dietary intake, the food content, the technical treatment of the products, the presence of substances that limit or increase the bioavailability of minerals and the physiological state of the food and overall health status of consumer.

The importance of optimal intakes of essential nutrient to maintain peak health is widely recognized (Avioli, 1988). Optimal intakes of elements such as sodium, potassium, magnesium, calcium, manganese, copper, zinc, and iodine could reduce individual risk factors, including those related to cardiovascular disease (Mertz, 1982). It also has been recognized that some elements such as selenium could play a protective role in decreasing the risk of some types of cancer (World Cancer Report, 1997). Thus, balance diet rich in minerals, fiber and vitamins are more than perfect for the human health.

1.3 Antioxidant activity

1.3.1 Oxidative damage and diseases

Free radicals and other reactive species are waste products present in the body and can be generated endogenously and exogenously (Gaté *et al*., 1999). Unhealthy human diet containing mutagenic and carcinogenic substances and pathologic cell metabolism also contribute to the formation of free radicals.

Free radicals are atom, molecule or mixture containing one or more unpaired electron (Forrester *et al*., 1968). The species are capable to extract electron from other molecules to stabilize the electron number, thus led to the formation of new free radicals known as reactive oxygen species (ROS) (Stengler, 2001). Various ROS such as singlet oxygen ($^1O_2$), superoxide radical ($O^-_2\bullet$), hydroxyl radical ($OH\bullet$) and hydrogen peroxide (H_2O_2) are generated as by - products during aerobic metabolism in cells (Gutteridge, 1994), which have the potential for bringing about extensive damages to living cells (Darley - Usmar *et al.*, 1995).

Within the cells, ROS will enter intercellular space and subsequently attack oxidizable substrates such as DNA, lipids, proteins and carbohydrates (Halliwell, 1995). These will cause DNA lesions, lipid peroxidation, protein fragmentation within the cells of biological macromolecules (Gutteridge, 1994) and subsequently destroy membrane integrity and resulting cell lyses (Wei & Shiow, 2001). Vast scientific reports acknowledged that the oxidative stress is an important contributor to the pathophysiology of a variety of pathological conditions including cardiovascular dysfunctions, atherosclerosis, carcinogenesis, inflammation, neurodegenerative diseases such as such as Alzheimer's disease, Parkinson's disease and Downs syndrome (Manach *et al.*, 2004) and in natural aging processes (Govindarajan *et al*., 2005).

1.3.2 Phenolic compounds as natural antioxidant

Phenolic substances are secondary metabolites compounds synthesized by plant and derived from phenylalanine and tyrosine pathways (Shahidi & Naczk, 2004). Plant phenolics include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoid, stilbenes, hydrolysable and condensed tannins, lignins and lignans. Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents and range from simple phenolic molecules to highly polymerized compounds (Sakihama *et al*., 2002). These substances may act as phytoalexins, pigments, antioxidants, attractant for pollinators and protective agents against UV light (Harborne, 1967; McClure, 1975; Timberlake & Bridle, 1975; Heim *et al*., 2002). In food, phenolic substances may contribute to the bitterness, astringency, flavor, odor and color (Shahidi & Naczk, 1995). Phenolic compounds are not uniformly distributed in plant at the tissue, cellular and subcellular levels (Maisuthisakul *et al*., 2008). The content in plants are differed due to genetic and environmental factors as well as post - harvest and storage conditions (Franke *et al*., 2004).

Halliwell (1995) defined antioxidant as a substance that, when present at low concentrations compared to substrate, significantly inhibit or delay the oxidation of substrates by inhibiting the initial or propagation of oxidation chain reactions.

Antioxidants are actively involved in preventing free radical damage (Seifried *et al*., 2007). Antioxidants are divided into two main types according to their action. Primary antioxidants can inhibit or delay oxidation by scavenging reactive oxygen species. Secondary antioxidants function by binding metal ions, converting hydroperoxides to non - radical species, absorbing UV radiations or deactivating singlet oxygen (Gordon, 2001). Antioxidants are also believed to contribute to the beneficial effects through stimulating the antioxidative defense enzyme activities (Saha *et al*., 2004).

Among the various kinds of natural antioxidants, polyphenols have received much attention (Luo *et al*., 2002). The ability of natural phenolic substances including flavonoids and phenolic acids as antioxidant agents has been extensively investigated (Rice - Evans *et al*., 1996; Shui & Lai, 2004; Kim *et al*., 2005; Duarte - Almeida *et al*., 2007). Phenolic antioxidants in plants tend to be water soluble and most of them appear as glycosides and they are located in the cell vacuoles (Harborne, 1998). Dietary consumption of fruits and vegetables contain abundant of natural ROS scavenging molecules including phenolic compounds (Shahidi & Naczk, 1995; Aruoma, 2003). These exogenous antioxidants are required to maintain adequate level of antioxidants in human body for disease prevention and health promotion. These natural diets tend to be safer than synthetic antioxidant such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), which are extensively used in food processing industry (Mathew & Abraham, 2006).

Among the phenolic compounds, flavonoids are the most well - known antioxidant. Phenolic structure provided a primer factor of antioxidant property (Rice - Evans &

Miller, 1998). The basic flavonoids structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6 - C3 - C6), labeled A, B and C. Vary in plant antioxidant properties mostly due to hydroxylation, glycosylation, aromatic substitution and conjugation with phenolic compounds or organic acid (Heim *et al*., 2002). In addition to antioxidant properties of natural flavonoids, extensive investigation has been done to reveal the pharmacological aspects such as antiallergic, antiatherogenic, antiinflammatory, antimicrobial, antithrombotic, cardioprotective and vasodilatory effects (Balasundram *et al*., 2006).

Natural phenolic antioxidants (NPH) hinder the oxidation process of substrates by rapid donation of a hydrogen atom to radicals (RO●).

$$RO\bullet + NPH \rightarrow NP\bullet + ROH$$

A stable intermediate substance, phenoxy radical (NP●) act as terminators of the propagation route by reacting with other free radicals (Ferguson, 2001).

$$RO\bullet + NP\bullet \rightarrow RONP$$

1.4 Antibacterial activity

1.4.1 Diseases and antibacterial agents

Throughout history, there has been a continual battle between humans and the multitude of microorganisms that cause various types of infections and diseases. A variety of microorganisms may also lead to food spoilage that will threaten for both consumers and the food industries.

Cheesbrough (1984) defined antibacterial agents as any chemical substances that, when present at certain concentration are capable to kill or inhibit the growth of bacteria. Bacteriostatic agents prevent the growth of bacteria while bactericidal agents are capable to kill the bacteria (Nester *et al*., 2007). Action of antibacterial agents falls into four general categories; through inhibition of cell wall, protein and nucleic acid synthesis or by disturbing the cell membrane function (Talaro & Talaro, 2002).

Multiple drug resistance has become a global concern (Westh *et al*., 2004) due to indiscriminate use of commercial antimicrobial drugs in the treatment of infectious diseases (Service, 1995). In a recent study done in New York City, up to 50% of *Streptococcus pneumonia* isolates obtained from two institutions were resistant to erythromycin (Lin *et al*., 2004). Resistance among bacteria genera are due to, first, by prevention of interaction of drugs with target, secondly by effluxing of the antibacterial agent from the cell and lastly, by modification of bacteria's structure (Mendonco - Filho, 2006).

Numerous studies have identified compounds within plants that are effective antibacterial (Basile *et al*., 1997; Cowan, 1999; Basile *et al*., 2000; Mackeen *et al*., 2000). Herbal remedies utilized in traditional healing systems around the world are important resources for the discovery of new antibacterial compounds (Okpekon *et al*., 2004).

1.4.2 Roles of phenolic compounds in treating bacterial diseases

Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from natural sources. However, the development of bacterial resistance to the available known antibiotics, the emergence of uncommon infections (Liu *et al*., 2008), the undesirable side effects of certain antibiotics such as hypersensitivity, immunosuppression and allergic (Ahmad *et al*., 1998; Sudha *et al*., 2001) and the acceptance of traditional medicine as an alternative form for health care have led researchers to investigate the antibacterial activity of medicinal plants (Mahasneh & El - Oqlah, 1999; Sahin *et al*., 2003; Baydar *et al*., 2004; Venkat Reddy *et al*, 2004; Loziene *et al*., 2007; Adedapo *et al*., 2008). According to Atlas (1984), the commercialization of antibacterial agents as chemotherapeutic drugs is influenced by several factors such as solubility, stability, excretion rate, allergic reaction and toxicity to cell.

The use of complementary and alternative medicine has increased dramatically. This situation forced the scientists to validate the therapeutic values of various sources medicinal plants thus ascertain new antimicrobial substances. For instance, antibacterial activity of common herbal remedies of Texas: *Achillea millefolium*, *Berberis vulgaris*, *Commiphora molmol*, *Galium aparine*, *Glycyrrhiza glabra*, *Matricaria chamomilla*, *Pimenta dioica*, *Salvia greggii*, *Uncaria tomentosa* and *Zea mays* were reported by Romero *et al.* (2005). Mothana and Lindequist (2005) have screened the antimicrobial activity of extracts of 25 selected plants belonging to 19 families from the island Soqotra against several bacteria including *Staphylococcus* strains. The results revealed the potential value of *Punica protopunica*, *Boswellia* species, *Commiphora parvifolia*, *Buxus hildebrandtii*, *Jatropha unicostata*, *Kalanchoe farinacea* and *Withania* species as antibacterial drugs against Gram - positive bacteria.

Among the various kinds of secondary metabolites in plants polyphenols have received much attention as antibacterial agents (Karamanoli, 2002). Extracts of various medicinal plants containing flavonoids have been reported to possess antimicrobial activity (El - Abyad *et al*., 1990; Singh & Nath, 1999; Cakir *et al*., 2003; Sato *et al*., 2004). For centuries, preparations containing phenolic compounds as the active constituents have been utilized by physicians and lay healers in attempt to treat infectiuos diseases (Havsteen, 1983). Huang chin (*Scutellaria baicalensis*) is yet a good example. This herb is believed for many thousands of years in China for the treatment of periodontal abscesses and infected oral wounds, by applying systemically and topically. A flavone, baicalein was reported as the antibacterial compound of this plant (Tsao *et al*., 1982). A list of plants with antibacterial phenolic compounds and its mechanism of action are shown in Table 1.2.

Table 1.2 Phenolic antibacterial compounds and their mechanism of action (Cushnie & Lamb, 2005)

	Mechanism of action
1) Inhibition of nucleic acid synthesis	
Phenolic compounds	
Robinetin, myricetin and (-) - epigallocatechin	Inhibit DNA and RNA synthesis
Quercetin, apigenin and 3, 6, 7, 3', 4' - Pentahydroxyflavone	Inhibit DNA gyrase
Rutin	Promote topoisomerase IV - dependent DNA cleavage Inhibit topoisomerase IV - dependent decatenation activity
Galangin	Topoisomerase IV and the relatively homologous gyrase enzyme are involved
2) Inhibition of cytoplasmic membrane function	
Phenolic compounds	
Sophoraflavanone G and naringenin	Alteration of membrane fluidity in hydrophilic and hydrophobic regions thus reduced the fluidity of outer and inner layers of membranes
(-) - epigallocatechin gallate	Perturb the lipid bilayers by directly penetrating them and disrupting the barrier function membrane fusion, a process that results in leakage of intramembranous materials and aggregation
(-) - epicatechin gallate and 3 - *O* - octanoyl - (+) – catechin	Act on and damage bacterial membrane
2, 4, 2' - trihydroxy - 5' - methylchalcone	Change the permeability of the cellular membrane and damaging membrane function
Galangin	Induces cytoplasmic membrane damage and potassium leakage
Naringenin and quercetin	Increase in permeability of the inner bacterial membrane and a dissipation of the membrane potential inhibited bacterial motility
3) Inhibition of energy metabolism	
Phenolic compounds	
Retrochalcones (licochalcone A and C)	Interfering with energy metabolism, Inhibit oxygen consumption and NADH - cytochrome c reductase
Lonchocarpol A	Interferes with energy metabolism

The antimicrobial mode of action is related with the phenolic compounds (Cakir *et al*., 2004). It is also worth determining the antibacterial mechanism of action of various phenolic compounds. Phenolics attack and disturb the structure of lipid bilayers membrane by penetrating into them and disturbing the barrier function. This may cause membrane fusion, a process that results in leakage of intramembranous materials and aggregation (Ikigai *et al*., 1993), capable to change the permeability of the cellular membrane and damaging membrane potential (Sato *et al*., 1997). They also interfere with membrane function via electron transport, nutrient uptake, protein and nucleic acid synthesis and enzyme activity (Denyer & Hugo, 1991). It is also believed that chelation of transition reactive metals ions, such as iron and copper, by phenolic compounds reduces bioavailability for bacterial growth (Jay, 1996).

1.5 Problem statement

None of the previous studies were ever highlighted on the physicochemical characteristic including color parameter, proximate and mineral analyses of different parts of *M. fragrans*. Even though many scientific studies have been conducted on this plant, more emphasis was given to mace, seed and seed kernel extracts. There have been no attempts to verify the therapeutic values of leaf, pericarp and shell extracts. Comparative evaluation between extracts of different plant parts is also lacking.

Due to the limited data, the aim of this study is to provide new information on the physicochemical profiling, phenolic content, antioxidant activity and antibacterial activity of the plant.

1.6 Objectives of study

The aims of the present study are as follows:

1. To quantify and compare the physicochemical properties of six parts of *M. fragrans*.
2. To determine and compare the total phenolic content, free radical scavenging activity and the antibacterial property of the extracts.
3. To correlate between total phenolic content and free radical scavenging activity of the extracts.
4. To detect the active antioxidant and antibacterial compounds.

CHAPTER 2 LITERATURE REVIEW

2.1 Physicochemical profiling of *M. fragrans*

Limited data is available on physicochemical properties of *M. fragrans*. The only data is on the general composition of seed kernel and mace that was obtained from Gopalakrishnan (1992) and is presented in Table 2.1.

Table 2.1 Analysis on *M. fragrans* (Gopalakrishnan, 1992)

Composition	**Plant part**	
	Seed kernel (%)	**Mace (%)**
Moisture	40.00	40.00
Volatile oil	11.00	15.30
Non - volatile oil ether extract	33.60	21.98
Starch	30.20	44.05
Sugar		
Glucose	0.10	0.17
Fructose	0.07	0.10
Total reducing sugars	0.17	0.27
Sucrose	0.72	0.39
Total sugars	0.89	0.65
Protein	7.16	9.91
Crude fiber	11.70	3.93
Total ash	2.57	1.56
Ash insoluble in HCl	0.20	0.15
Polyphenols		
Total tannins	2.50	-
True tannins	1.00	-

Based on the data in Table 2.1, the moisture content level of the seed kernel and mace were equal (40.00%). In mace, the amounts of volatile oil, starch, glucose, fructose, total reducing sugars, sucrose and protein were higher than that of the seed kernel. The seed kernel has higher amount of non - volatile oil ether extract, sucrose, total sugars, crude fiber, total ash and ash insoluble in acid hydrochloric (HCl). Polyphenol was only detected in seed kernel (2.50% of total tannin and 1.00% of true tannins).

2.2 Phenolic compounds of *M. fragrans*

Phenolic substances are ubiquitously distributed throughout the plant kingdom especially in fruits and vegetables. Several studies have quantified the total phenolic content in pericarp, mace and seed kernel of *M. fragrans*. The results are summarized in Table 2.2. However, the result cannot be compared among one another due to the different in extraction procedures and phenolic content estimation protocols.

Various phenolic compounds have been isolated from *M. fragrans*. According to a review by Suhaj (2006), quercetin and kaempferol were widely distributed in leaf, pericarp, mace and seed. On the other hand, Shan *et al*. (2005) have detected the presence of caffeic acid and catechin in the pericarp. Ferulic acid was also found in the pericarp (Wojdylo *et al*., 2007). Hada *et al*. (1988) have isolated eight neolignans and five lignans from the mace part. Kwon *et al*. (2008) reported the presence of six diarylbutane lignans and one aryltetralin lignan in the 95% methanol extracts of the seeds and 7 - methyl ether diarylbutane lignan was a novel compound. Li and Yang (2008) found that myrislignan is a major acyclic neolignan in seeds.

Table 2.2 Total phenolic content of *M. fragrans*

Plant part	Extract	Total phenolic content	References
Pericarp	80% methanol	1.61 ± 0.00g GAE/100g of dry weight	Shan *et al*. (2005)
	80% methanol	8.95 ± 0.45mg GAE/100g of dry weight	Wojdylo *et al*. (2007)
Mace	Acetone	40mg CE/100g of fresh weight	Chatterjee *et al*., (2007)
	80% methanol	1.98g GAE/100g dry weight	Surveswaran *et al*. (2007)
Seed kernel	80% methanol	1.30g GAE/100g dry weight	Surveswaran *et al*. (2007)
	80% methanol and 50% acetone	2.68 ± 0.120mg GAE/g extract for 50% acetone extract, 2.62 ± 0.01mg GAE/g extract for 80% methanol extract	Su *et al*. (2007)
	Methanol	153.00 ± 1.00mg GAE/g dry weight	Ho *et al*. (2008)

Data expressed as gallic acid equivalents (GAE) or catechin equivalent (CE).

2.3 Antioxidant properties of *M. fragrans*

Various researches were done to determine the antioxidant properties of the *M. fragrans*. The results were compiled in Table 2.3. Several assays were used such as 2, 2' - azino - bis - 3 - ethyl benzthiazoline - 6 - sulfonic acid (ABTS●$^+$) and 1, 1 - diphenyl - 2 - picryl hydrazyl (DPPH●) radical scavenging activities, ferric reducing/ antioxidant power (FRAP), ferric thiocyanate (FTC), thiobarbituric acid (TBA), *in vitro* thiobarbituric acid reactive substances (TBARS) and oxygen radical absorbance capacity (ORAC). The 2, 2' - bipyridyl competition assay was also conducted to measure the Fe^{2+} - chelating activity and hydroxyl radical (HO●) - scavenging capacity was examined by the electron spin resonance (ESR) spectroscopy method.

The result obtained from FRAP and ABTS●$^+$ assay were categorized into five main groups. The sample with trolox equivalent antioxidant capacity (TEAC) over 500µM/100g is classified as containing extremely high activity, from 100 to 500µM/100g, 50 to 100µM/100g and 10 to 50µM/100g and less than 10µM/100g were classified as containing high, good, low and very low antioxidant capacities, respectively. Thus, the outcomes in Table 2.3 were discussed based on these categories.

Table 2.3 Antioxidant evaluation of *M. fragrans*

Plant part	Extract	Antioxidant test	Outcome	References
Pericarp	80% methanol	ABTS●$^+$	Extract has low antioxidant activity	Shan *et al*. (2005)
	80% methanol	FRAP, ABTS●$^+$ and DPPH●$^+$	Extract has a moderate antioxidant values in ABTS●$^+$ and DPPH●$^+$ assay and high antioxidant capacity in FRAP test	Wojdylo *et al*. (2007)
Mace	Acetone	FTC, TBA and DPPH●$^+$	Extract and essential oil showed stronger activity than BHA and BHT	Singh *et al*. (2005)
	Acetone	DPPH●$^+$ and β - carotene - linoleic acid	Extract showed better radical scavenging activity than its three lignan fractions and these fractions were capable to inhibit peroxidation	Chatterjee *et al*. (2007)
	80% methanol	ABTS●$^+$, DPPH●$^+$ and FRAP	Extract was considered having good antioxidant capacity	Surveswaran *et al*. (2007)
Seed	95% methanol	TBRAS	Extract has the ability to protect human LDL against Cu^{2+}induced peroxidation	Kwon *et al*. (2008)
Seed kernel	80% methanol and 50% acetone	ABTS●$^+$, DPPH●$^+$ 2, 2' - bipyridyl competition assay, ESR and ORAC	50% acetone extract has lower EC_{50} value, higher chelating activity against Fe^{2+} and scavenging activity on HO● than 80% methanol extract. 80% methanol extract has greater ABTS●$^+$ and ORAC value than 50% acetone extract	Su *et al*. (2007)
	80% methanol	ABTS●$^+$, DPPH●$^+$ and FRAP	Extract was considered having good antioxidant capacity	Surveswaran *et al*. (2007)
	Methanol	ABTS●$^+$, DPPH●$^+$ and ORAC	Extract was considered having good antioxidant activity	Ho *et al*. (2008)

As indicated in Table 2.3, Surveswaran *et al*. (2007) have compared the antioxidant activities of the 80% methanol extract of the mace and seed kernel through ABTS$\bullet^{+}$, DPPH$\bullet$ and FRAP assays. It was found that the TEAC value of ABTS$\bullet^{+}$ assay of the mace was higher than the seed kernel extracts with 26.03mmol trolox per 100g dry weight (mmol trolox/100g) and 17.92mmol trolox/100g, respectively. Moreover, the seed kernel (13.31mmol trolox/100g) was highly capable to scavenge DPPH free radical as compared to mace extract (9.70mmol trolox/100g). The authors also found that the 80% methanol extract of both parts exhibited more or less similar FRAP capacity.

2.4 Antibacterial properties of *M. fragrans*

Based on anti - *Helicobacter pylori* comparative evaluation of various Thai medicinal plants, *M. fragrans* mace extract gave the lowest minimum inhibitory concentration (MIC) that was 12.50µg/mL (Bhamarapravati *et al*., 2003). The leaf extract also has low MIC of 50.00µg/mL. Zaidi *et al*. (2009) found that 70% ethanol extract of mace (minimum bactericidal concentration (MBC) value ranged from 62.50µg/mL to 31.20µg/mL) showed stronger bactericidal activity than 70% ethanol extract of seed (MBC value ranged from 125.00µg/mL to 62.50µg/mL).

Screening by Rani and Khullar (2004) on some traditional Ayurvedic medicine against resistant *Salmonella thypi*, resulted a strong antibacterial activity of the methanol extract of the seed. Consequently, Mahady and colleagues (2005) found that the MIC value of the methanol extract of the seed was 12.50µg/mL against *Helicobacter pylori*. Chung *et al*. (2006) have investigated the antibacterial activities